

SPECIFICATION

Title of the Invention

Process for Producing Microbial Transglutaminase

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Background of the Invention

The present invention relates to a protein having a transglutaminase activity, DNA which encodes for the protein, and a process for producing the protein. In particularly, the present invention relates to a process for producing a protein having a transglutaminase activity by a genetic engineering technique.

Transglutaminase is an enzyme which catalyzes the acyl transfer reaction of a γ -carboxyamido group in a peptide chain of a protein. When such an enzyme react with the protein, a reaction of an $\epsilon-(\gamma-\text{Glu})$ -Lys forming reaction or substitution reaction of Gln with Glu by the deamidation of Glu can occur.

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The transglutaminase is used for the production of gelled foods such as jellies, yogurts, cheeses, gelled cosmetics, etc. and also for improving the quality of meats [see Japanese Patent Publication for Opposition Purpose (hereinafter referred to as "J. P. KOKOKU") No. Hei 1-50382]. The transglutaminase is also used for the production of a material for microcapsules having a high thermal stability and a carrier for an immobilized enzyme. The transglutaminase is thus industrially very useful.

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As for transglutaminases, those derived from animals and those derived from microorganisms (microbial transglutaminase; hereinafter

referred to as "MTG") have been known hitherto.

The transglutaminases derived from animals are calcium ion-dependent enzymes which are distributed in organs, skins and bloods of animals. They are, for example, guinea pig liver transglutaminase [K.Ikura et al., Biochemistry 27, 2898 (1988)], human epidermis keratin cell transglutaminase [M. A. Philips et al., Proc. Natl. Acad. Sci. USA 87, 9333 (1990)] and human blood coagulation factor XIII (A. Ichinose et al., Biochemistry 25, 6900 (1990)].

As for the transglutaminases derived from microorganisms, those independent on calcium were obtained from microorganisms of the genus Streptoverticillium. They are, for example, Streptoverticillium griseocarneum IFO 12776, Streptoverticillium cinnamoneum sub sp. cinnamoneum IFO 12852 and Streptoverticillium moharaense IFO 13819 [see Japanese Patent Unexamined Published Application (hereinafter referred to as "J. P. KOKAI") No. Sho 64-27471].

According to the peptide mapping and the results of the analysis of the gene structure, it was found that the primary structure of the transglutaminase produced by the microorganism is not homology with that derived from the animals at all (European Patent publication No. 0 481 504 A1).

Since the transglutaminases (MTG) derived from microorganisms are produced by the culture of the above-described microorganisms followed by the purification, they had problems in the supply amount, efficiency, and the like. It is also tried to produce them by a genetic engineering technique. This technique includes a process which is conducted by the secretion expression of a microorganism such as E.

coli, yeast or the like (J. P. KOKAI No. Hei 5-199883), and a process wherein MTG is expressed as an inactive fusion protein inclusion body in E. coli, this inclusion body is solubilized with a protein denaturant, the denaturant is removed and then MTG is reactivated to obtain the active MTG (J. P. KOKAI No. Hei 6-30771).

5 However, these processes have problems when they are practiced on an industrial scale. Namely, when the secretion by the microorganisms such as E. coli and yeast is employed, the amount of the product is very small; and when MTG is obtained in the form of the inactive fusion protein inclusion body in E. coli, an expensive enzyme is necessitated for the cleavage.

10 It is known that when a foreign protein is secreted by the genetic engineering method, the amount thereof thus obtained is usually small. On the contrary, it is also known that when the foreign protein is produced in the cell of E. coli, the product is in the form of inert protein inclusion body in many cases although the expressed amount is high. The protein inclusion body must be solubilized with a denaturant, the denaturating agent must be removed and then MTG must be reactivated.

15 It is already known that in the expression in E. coli, an N-terminal methionine residue in natural protein obtained after the translation of gene is efficiently cleaved with methionine aminopeptidase. However, the N-terminal methionine residue is not always cleaved in an exogenous protein.

20 Processes proposed hitherto for obtaining a protein free from N-terminal methionine residue include a chemical process wherein a protein

having methionine residue at the N-terminal or a fusion protein having a peptide added thereto through methionine residue is produced and then the product is specifically decomposed at the position of methionine residue with cyanogen bromide; and an enzymatic process wherein a 5 recognition sequence of a certain site-specific proteolytic enzyme is inserted between a suitable peptide and an intended peptide to obtain a fusion peptide, and the site-specific hydrolysis is conducted with the enzyme.

However, the former process cannot be employed when the protein 10 sequence contains a methionine residue, and the intended protein might be denatured in the course of the reaction. The latter process cannot be employed when a sequence which is easily broken down is contained in the protein sequence because the yield of the intended protein is reduced. In addition, the use of such a proteolytic enzyme is 15 unsuitable for the production of protein on an industrial scale from the viewpoint of the cost.

Conventional processes for producing MTG have many problems such 20 as supply amount and cost. Namely, in the secretion expression by *E. coli*, yeast or the like, the expressed amount is disadvantageously very small. In the production of the fusion protein inclusion body in *E. coli*, it is necessary, for obtaining mature MTG, to cleave the fusion part with restriction protease after the expression. Further, it has been found that since MTG is independent on calcium, the expression of 25 active MTG in the cell of a microorganism is fatal because this enzyme acts on the endoprotein.

Thus, for the utilization of MTG, produced by the gene

recombination, on an industrial scale, it is demanded to increase the production of mature MTG free of the fusion part. The present invention has been completed for this purpose. The object of the present invention is to product MTG in a large amount in microorganisms such as E. coli.

When MTG is expressed with recombinant DNA of the present invention, methionine residue is added to the N-terminal of MTG. However, by the addition of the methionine residue to the N-terminal of MTG, there is some possibility wherein problems of the safety such as impartation of antigenicity to MTG occur. It is another problem to be solved by the present invention to produce MTG free of methionine residue corresponding to the initiation codon.

Summary of the Invention

An object of the present invention is to provide a novel protein having a transglutaminase activity.

Another object of the present invention is to provide a DNA encoding for the novel protein having a transglutaminase activity.

Another object of the present invention is to provide a recombinant DNA encoding for the novel protein having a transglutaminase activity.

Another object of the present invention is to provide a transformant obtained by the transformation with the recombinant DNA.

Another object of the present invention is to provide a process for producing a protein having a transglutaminase activity.

These and other objects of the present invention will be

apparent from the following description and examples.

For solving the above-described problems, the inventors have constructed a massive expression system of protein having transglutaminase activity by changing the codon to that for E. coli, or preferably by using a multi-copy vector (pUC19) and a strong promoter (trp promoter).

Since MTG is expressed and secreted in the prepro-form from microorganisms of actinomycetes, the MTG does not have methionine residue corresponding to the initiation codon at the N-terminal, but the protein expressed by the above-described expression method has the methionine residue at the N-terminal thereof. To solve this problem, the inventors have paid attention to the substrate specificity of methionine aminopeptidase of E. coli, and succeeded in obtaining a protein having transglutaminase activity and free from methionine at the N-terminal by expressing the protein in the form free from the aspartic acid residue which is the N-terminal amino acid of MTG. The present invention has been thus completed.

Namely, the present invention provides a protein having a transglutaminase activity, which comprises a sequence ranging from serine residue at the second position to proline residue at the 331st position in an amino acid sequence represented by SEQ ID No. 1 wherein N-terminal amino acid of the protein corresponds to serine residue at the second position of SEQ ID No. 1.

There is provided a protein which consists of an amino acid sequence of from serine residue at the second position to proline residue at the 331st position in an amino acid sequence of SEQ ID No. 1.

There is provided a DNA which codes for said proteins.

There is provided a recombinant DNA having said DNA, in particular, a recombinant DNA expressing said DNA.

5 There is provided a transformant obtained by the transformation

with the recombinant DNA.

There is provided a process for producing a protein having a transglutaminase activity, which comprises the steps of culturing the transformant in a medium to produce the protein having a transglutaminase activity and recovering the protein.

10 Taking the substrate specificity of methionine aminopeptidase into consideration, the process for producing the protein having transglutaminase activity and free of initial methionine is not limited to the removal of the N-terminal aspartic acid.

15 Brief Explanation of the Drawings

Fig. 1 shows a construction scheme of MTG expression plasmid pTRPMTG-01.

Fig. 2 shows a construction scheme of MTG expression plasmid pTRPMTG-02.

20 Fig. 3 is an expansion of SDS-polyacrylamide electrophoresis showing that MTG was expressed.

Fig. 4 shows a construction scheme of MTG expression plasmid pTRPMTG-00.

Fig. 5 shows a construction scheme of plasmid pUCN216D.

25 Fig. 6 shows a construction scheme of MTG expression plasmid PUCTRPMTG(+)D2.

Fig. 7 shows that GAT corresponding to Aspartic acid residue is deleted.

Fig. 8 shows that N-terminal amino acid is serine.

5 Detailed Description of the Preferred Embodiments

The proteins having a transglutaminase activity according to the present invention comprise a sequence ranging from serine residue at the second position to proline residue at the 331st position in an amino acid sequence represented by SEQ ID No. 1 as an essential sequence but the protein may further have an amino acid or amino acids after proline residue at the 331st position. Among these, the preferred is a protein consisting of an amino acid sequence of from serine residue at the second position to proline residue at the 331st position in an amino acid sequence of SEQ ID No. 1.

In these amino acid sequences, the present invention includes amino acid sequences wherein an amino acid or some amino acids are deleted, substituted or inserted as far as such amino acid sequences have a transglutaminase activity.

The DNA of the present invention encodes the above-mentioned proteins. Among these, the preferred is a DNA wherein a base sequence encoding for Arg at the forth position from the N-terminal amino acid is CGT or CGC, and a base sequence encoding for Val at the fifth position from the N-terminal amino acid is GTT or GTA. Furthermore, the preferred is a DNA wherein a base sequence encoding for the N-terminal amino acid to fifth amino acid, Ser-Asp-Asp-Arg-Val, has the following sequence.

Ser : TCT or TCC

Asp : GAC or GAT

Asp : GAC or GAT

Arg : CGT or CGC

5 Val : GTT or GTA

In this case, the preferred is a DNA wherein a base sequence encoding for amino acid sequence of from the N-terminal amino acid to fifth amino acid, Ser-Asp-Asp-Arg-Val, has the sequence TCT-GAC-GAT-CGT-GTT.

Furthermore, the preferred is a DNA wherein a base sequence encoding for amino acid sequence of from sixth amino acid to ninth amino acid from the N-terminal amino acid, Thr-Pro-Pro-Ala, has the following sequence.

10 Thr : ACT or ACC

Pro : CCA or CCG

15 Pro : CCA or CCG

Ala : GCT or GCA

Furthermore, the preferred is a DNA comprising a sequence ranging from thymine base at the fourth position to guanine base at the 993rd position in the base sequence of SEQ ID No. 2. In this case, more preferred is a DNA consisting of a sequence ranging from thymine base at the fourth position to guanine base at the 993rd position in the base sequence of SEQ ID No. 2.

20 In the DNA sequences mentioned above, nucleic acids encoding an amino acid or some amino acids may be deleted, substituted or inserted as far as such DNA encodes an amino acid sequence having a transglutaminase activity.

The recombinant DNA of the present invention has one of DNA mentioned above. In this case, the preferred is a DNA having a promoter selected from the group consisting of trp, tac, lac, trc, λ PL and T7.

5 The transformants of the present invention are obtained by the transformation with the above-mentioned recombinant DNA. Among these, it is preferable that a transformation be conducted by use of a multi-copy vector, and that the transformants belong to Escherichia coli.

10 The process for producing a protein having a transglutaminase activity according to the present invention comprises the steps of culturing one of the above-mentioned transformants in a medium to produce the protein having a transglutaminase activity and recovering the protein.

15 The detailed description will be further made on the present invention.

(1) It is known that the expression of MTG in the cells of a microorganism is fatal. It is also known that in the high expression of the protein in a microorganism such as E. coli, the expressed protein is inclined to be in the form of inert insoluble protein inclusion bodies. Under these circumstances, the inventors made investigations 20 for the purpose of obtaining a high expression of MTG as an inert, insoluble protein in E. coli.

25 A structural gene of MTG used for achieving the high expression is a DNA containing a sequence ranging from thymine base at the fourth position to guanine base at the 993rd position in the base sequence of SEQ ID No. 2. Taking the degeneration of the genetic codon, the third letter in the degenerate codon in a domain which codes for the N-

terminal portion is converted to a codon rich in adenine and uracil and the remaining portion is comprised of a codon frequently used for E. coli in order to inhibit the formation of high-order structure of mRNA, though a DNA which codes for proteins having the same amino acid sequence can have various base sequences.

A strong promoter usually used for the production of foreign proteins is used for the expression of MTG structural gene, and a terminator is inserted into the downstream of MTG structural gene. For example, the promoters are trp, tac, lac, trc, λ PL and T7, and the terminators are trpA, lpp and T4.

For the efficient translation, the variety and number in the SD sequence, and the base composition, sequence and length in the domain between the SD sequence and initiation codon were optimized for the expression of MTG.

The domain ranging from the promoter to the terminator necessitated for the expression of MTG can be produced by a well-known chemical synthesis method. An example of the base sequence is shown in SEQ ID No. 3. In the amino acid sequence of sequence No. 3, aspartic acid residue follows the initiation codon. However, this aspartic acid residue is preferably removed as will be described below.

The present invention also provides a recombinant DNA usable for the expression of MTG.

The recombinant DNA can be produced by inserting a DNA containing the structural gene of the above-described MTG in a known expression vector selected depending on a desired expression system. The expression vector used herein is preferably a multi-copy vector.

Known expression vectors usable for the production of the recombinant DNA of the present invention include pUC19 and pHSG299. An example of the recombinant DNA of the present invention obtained by integrating DNA of the present invention into pUC19 is pUCTRPMTG-02(+).

5 The present invention also relates to various transformants obtained by the introduction of the recombinant DNA.

The cells capable of forming the transformant include E. coli and the like.

An example of E. coli is the strain JM109 (recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, Δ (lac-proAB)/F' [traD36, proAB+, lacIq, lacZ Δ M15]).

A protein having a transglutaminase activity is produced by culturing the transformant such as that obtained by transforming E. coli JM109 with pUCTRPMTG-02(+) which is a vector of the present invention.

Examples of the medium used for the production include 2xYT medium used in the Example given below and medium usually used for culturing E. coli such as LB medium and M9-Casamino acid medium.

The culture conditions and production-inducing conditions are suitably selected depending on the kinds of the vector, promoter, host and the like. For example, for the production of a recombinant product with trp promoter, a chemical such as 3- β -indoleacrylic acid may be used for efficiently working the promoter. If necessary, glucose, Casamino acid or the like can be added in the course of the culture. Further, a chemical (ampicillin) resistant to genes which are resistant to chemicals kept in plasmid can also be added in order to selectively proliferate a recombinant E. coli.

The protein having a transglutaminase activity, which is produced by the above-described process, is extracted from the cultured strain as follows: After the completion of the culture, the cells are collected and suspended in a buffer solution. After the treatment with 5 lysozyme, freezing/melting, ultrasonic disintegration, etc., the thus-obtained suspension of the disintegrated cells is centrifuged to divide it into a supernatant liquid and precipitates.

The protein having a transglutaminase activity is obtained in the form of a protein inclusion body and contained in the precipitates. This protein is solubilized with a denaturant or the like, the denaturant is removed and the protein is separated and purified. Examples of the denaturants usable for solubilizing the protein inclusion body produced as described above include urea (such as 8M) and guanidine hydrochloride (such as 6 M). After removing the denaturant by the dialysis or the like, the protein having a transglutaminase activity is regenerated. Solutions used for the dialysis are a phosphoric acid buffer solution, tris hydrochloride buffer solution, etc. The denaturant can be removed not only by the dialysis but also dilution, ultrafiltration or the like. The regeneration of the 15 activity is expectable by any of these techniques.

After the regeneration of the activity, the active protein can be separated and purified by a suitable combination of well-known separation and precipitation methods such as salting out, dialysis, ultrafiltration, gel filtration, SDS-polyacrylamide electrophoresis, 20 ion exchange chromatography, affinity chromatography, reversed-phase high-performance liquid chromatography and isoelectric point

electrophoresis.

(2) The present invention provides a protein having a transglutaminase activity, which has a sequence ranging from serine residue at the second position to proline residue at the 331st position in the amino acid sequence represented in SEQ ID No. 1.

The N-terminals of MTG produced by the product transformed with recombinant DNA having a DNA represented in SEQ ID No. 3 was analyzed to find that most of them contained (formyl)methionine residue of the initiation codon.

However, when a gene which encodes for an exogenous protein is expressed in *E. coli*, the gene is designed so that the intended protein is positioned after the methionine residue encoded by ATG which is the translation initiation signal for the gene. It is already known that N-terminal methionine residues of a natural protein obtained by the translation from genes are more efficiently cut by methionine aminopeptidase. However, the N-terminal methionine residues are not always cut in the exogenous protein.

It is known that the substrate specificity of methionine aminopeptidase varies depending on the variety of the amino acid residue positioned next to the methionine residue. When the amino acid residue positioned next to the methionine residue is alanine residue, glycine residue, serine residue or the like, the methionine residue is easily cleaved, and when the former is aspartic acid, asparagine, lysine, arginine, leucine or the like, the latter is difficultly cleaved [Nature 326, 315(1987)].

The N-terminal amino acid residue of MTG is aspartic acid

residue. When a methionine residue derived from the initiation codon is positioned directly before the aspartic acid residue, methionine aminopeptidase difficultly acts on the obtained sequence, and the N-terminal methionine residue is usually not removed but remains. However,
5 since serine residue is arranged next to N-terminal aspartic acid in MTG, the sequence can be so designed that the amino acid residue positioned next to methionine residue derived from the initiation codon will be serine residue (an amino acid residue on which methionine aminopeptidase easily acts) by deleting aspartic acid residue. Thus, a
10 protein having a high transglutaminase activity, from which the N-terminal methionine residue has been cleaved, can be efficiently produced.

The recombinant protein thus obtained is shorter than natural MTG by one amino acid residue, but the function of this protein is the same as that of the natural MTG. Namely, MTG activity is not lost by the lack of one amino acid. Although there is a possibility that a protein having a transglutaminase activity, from which the methionine residue has not been cleaved, gains a new antigenicity, it is generally understood that the sequence shortened by several residues does not
15 gain a new antigenicity which natural MTG does not have. Thus, there
20 is no problem of the safety.

In fact, a sequence of Met-Ser-Asp-Asp-Arg- . . . was designed by deleting N-terminal aspartic acid residue from transglutaminase derived from microorganism (MTG), and this was produced
25 in E. coli. As a result, methionine residue was efficiently removed and thereby there was obtained a protein having a sequence of Ser-Asp-

Asp-Arg- It was confirmed that the specific activity of the thus-obtained protein is not different from that of natural MTG.

5 A process for producing a protein having a transglutaminase activity, which has a sequence ranging from serine residue at the second position to proline residue at the 331st position in the amino acid sequence represented in SEQ ID No. 1 will be described below.

That is, a DNA which encodes for a protein having a transglutaminase activity and having a sequence ranging from serine residue at the second position to proline residue at the 331st position in the amino acid sequence represented in SEQ ID No. 1 is employed as the MTG structural gene present on recombinant DNA usable for the expression of MTG. Concretely, a DNA having a sequence ranging from thymine base at the fourth position to guanine base at the 993rd position in the base sequence of SEQ ID No. 2 is employed.

15 The N-terminal sequence can be altered by an ordinary DNA recombination technique, or specific site directional mutagenesis technique, a technique wherein PCR is used for the whole or partial length of MTG gene, or a technique wherein the part of the sequence to be altered is exchanged with a synthetic DNA fragment by a restriction 20 enzyme treatment.

The transformant thus transformed with the recombinant DNA is cultured in a medium to produce a protein having a transglutaminase activity, and the protein is recovered. The methods for the preparation of the transformant and for the production of the protein are the same 25 as those described above.

Since the protein thus produced has a sequence of Met-Ser- . . .

• • from which the methionine residue is easily cleaved with methionine aminopeptidase, the methionine residue is cleaved in the cell of E. coli to obtain a protein that starts with serine residue.

Although MTG having N-terminal methionine residue is not present in the nature, the inventors have found that in some of natural MTG, aspartic acid residue is deleted to have N-terminal serine. Although a protein having N-terminal methionine residue is thus different from natural MTG in the sequence, a protein having N-terminal serine residue is included in the sequences of natural MTG and, in addition, a protein having such a sequence is actually present in the nature. Thus, it can be said that such MTG is equal to natural MTG. Namely, in the production of an enzyme to be used for foods, such as MTG, in which protein antigenicity is a serious problem, it is important to produce a protein having transglutaminase activity and also having a sequence equal to that of natural MTG, or in other words, to produce a sequence from which the N-terminal methionine residue was cleaved.

The following Examples will further illustrate the present invention, which by no means limit the invention.

Example

Mass production of MTG in E. coli:

<1> Construction of MTG expression plasmid pTRPMTG-01:

MTG gene has been already completely synthesized, taking the frequency of using codons of E. coli and yeast into consideration (J. P. KOKAI No. Hei 5-199883). However, the gene sequence thereof was not optimum for the expression in E. coli. Namely, all of codons of thirty arginine residues were AGA (minor codons). Under these conditions,

about 200 bases from the N-terminal of MTG gene were resynthesized to become a sequence optimum for the expression of E. coli.

As a promoter for transcribing MTG gene, trp promoter capable of easily deriving the transcription in a medium lacking tryptophane was used. Plasmid pTTG2-22 (J. P. KOKAI No. Hei 6-225775) for the high expression of transglutaminase (TG) gene of Pagrus major was obtained with trp promoter. The sequence in the upstream of the TG gene of Pagrus major was designed so that a foreign protein is highly expressed in E. col i.

In the construction of pTRPMTG-01, the DNA fragment from ClaI site in the downstream of trp promoter to BglII site in the downstream of Pagrus major's TG expression plasmid pTTG2-22 (J. P. KOKAI Hei 6-225775) was replaced with the ClaI/HpaI fragment of the synthetic DNA gene and the HpaI/BamHI fragment(small) of pGEM15BTG (J. P. KOKAI Hei 6-30771).

The ClaI/HpaI fragment of the Synthetic DNA gene has a base sequence from ClaI site in the downstream of trp promoter of pTTG2-22 to translation initiation codon, and 216 bases from the N-terminal of MTG gene. The base sequence in MTG structural gene was determined with reference to the frequency of using codon in E. coli so as to be optimum for the expression in E. coli. However, for avoiding the high-order structure of mRNA, the third letter of the degenerated codon in the domain of encoding the N-terminal part was converted to a codon rich in adenine and uracil so as to avoid the arrangement of the same bases as far as possible.

The ClaI/HpaI fragment of the Synthetic DNA gene was so designed

that it had EcoRI and HindIII sites at the terminal. The designed gene was divided into blocks each comprising about 40 to 50 bases so that the + chain and the - chain overlapped each other. Twelve DNA fragments corresponding to each sequence were synthesized (SEQ ID Nos. 4 to 15).

5 5' terminal of the synthetic DNA was phosphatized. Synthetic DNA fragments to be paired therewith were annealed, and they were connected with each other. After the acrylamide gel electrophoresis, the DNA fragments of an intended size was taken out and integrated in EcoRI/HindIII sites of pUC19. The sequence was confirmed and the correct one was named pUCN216. From the pUCN216, a ClaI/HpaI fragment (small) was taken out and used for the construction of pTRPMTG-01.

10 15 20 25 <2> Construction of MTG expression plasmid pTRPMTG-02:

Since E. coli JM109 keeping pTRPMTG-01 did not highly express MTG, parts (777 bases) other than the N-terminal altered parts of MTG gene were altered suitably for E. coli. Since it is difficult to synthesize 777 bases at the same time, the sequence was determined, taking the frequency of using codons in E. coli into consideration, and then four blocks (B1, 2, 3 and 4) therefor, each comprising about 200 bases, were synthesized. Each block was designed so that it had EcoRI/HindIII sites at the terminal. The designed gene was divided into blocks of about 40 to 50 bases so that the + chain and the - chain overlapped each other. Ten DNA fragments of the same sequence were synthesized for each block, and thus 40 blocks were synthesized in total (SEQ ID Nos. 16 to 55). 5' terminal of the synthetic DNA was phosphatized. Synthetic DNA fragments to be paired therewith were annealed, and they were connected with each other. After the

acrylamide gel electrophoresis, DNA of an intended size was taken out and integrated in EcoRI/HindIII sites of pUC19. The base sequence of each of them was confirmed and the correct ones were named pUCB1, B2, B3 and B4. As shown in Fig. 2, B1 was connected with B2, and B3 was connected with B4. By replacing a corresponding part of pTRPMTG-01 therewith, pTRPMTG-02 was constructed. The sequence of the high expression MTG gene present on pTRPMTG-02 is shown in SEQ ID No. 3.

5 <3> Construction of MTG expression plasmid pUCTRPMTG-02(+), (-):

10 Since *E. coli* JM109 which keeps the pTRPMTG-02 also did not highly express MTG, the plasmid was multi-copied. EcoO109I fragment (small) containing trp promoter of pTRPMTG-02 was smoothened and then integrated into HincII site of pUC19 which is a multi-copy plasmid. pUCTRPMTG-02(+) in which lacZ promoter and trp promoter were in the same direction, and pUCTRPMTG-02(-) in which they were in the opposite direction to each other were constructed.

15 <4> Expression of MTG:

20 *E. coli* JM109 transformed with pUCTRPMTG-02(+) and pUC19 was cultured by shaking in 3 ml of 2xYT medium containing 150 μ g/ml of ampicillin at 37°C for ten hours (pre-culture). 0.5 ml of the culture suspension was added to 50 ml of 2xYT medium containing 150 μ g/ml of ampicillin, and the shaking culture was conducted at 37°C for 20 hours.

25 The cells were collected from the culture suspension and broken by ultrasonic disintegration. The results of SDS-polyacrylamide electrophoresis of the whole fraction, and supernatant and precipitation fractions both obtained by the centrifugation are shown in Fig. 3. The high expression of the protein having a molecular weight

equal to that of MTG was recognized in the whole fraction of broken pUCTRPMTG-02(+)/JM109 cells and the precipitate fraction obtained by the centrifugation. It was confirmed by the western blotting that the protein was reactive with mice anti-MTG antibody. The expression of the protein was 500 to 600 mg/L. A sufficient, high expression was obtained even when 3- β -indole acrylic acid was not added to the production medium.

Further, the western blotting was conducted with MTG antibody against mouse to find that MTG was expressed only slightly in the supernatant fraction obtained by the centrifugation and that the expressed MTG was substantially all in the form of insoluble protein inclusion bodies.

<5> Construction of MTG expression plasmid pTRPMTG-00:

To prove that the change in codon of MTG gene caused a remarkable increase in the expression, pTRPMTG-00 corresponding to pTRPMTG-02 but in which MTG gene was changed to a gene sequence completely synthesized before (J. P. KOKAI No. Hei 6-30771) was constructed.

pTRPMTG-00 was constructed by connecting Pvull/PstI fragment (small) from *Pagrus major*'s TG expression plasmid pTRPMTG-02 with PstI/HindIII fragment (small, including Pvull site) and Pvull/HindIII fragment (small) of pGEM15BTG (J. P. KOKAI No. Hei 6-30771).

<6> Construction of MTG expression plasmid pUCTRPMTG-00(+), (-):

pTRPMTG-00 was multi-copied. EcoO109I fragment (small) containing trp promoter and trpA terminator of pTRPMTG-00 was smoothened and then integrated into HincII site of pUC19 which is a multi-copy

plasmid. pUCTRPM TG-00(+) in which lacZ promoter and trp promoter were in the same direction, and pUCTRPM TG-00(-) in which they were in the opposite direction to each other were constructed.

<7> Comparison of MTG expressions:

E. coli JM109 transformed with pUCTRPM TG-02 (+) or (-), pUCTRPM TG-00 (+) or (-), pTRPM TG-02, pTRPM TG-01, pTRPM TG-00 or pUC19 was cultured by shaking in 3 ml of 2xYT medium containing 150 μ g/ml of ampicillin at 37°C for ten hours (pre-culture). 0.5 ml of the culture suspension was added to 50 ml of 2xYT medium containing 150 μ g/ml of ampicillin, and the shaking culture was conducted at 37 °C for 20 hours.

The cells were collected from the culture suspension, and MTG expression thereof was determined to obtain the results shown in Table 1. It was found that the newly constructed E. coli containing pTRPM TG-00, pUCTRPM TG-00 (+) or (-) did not highly express MTG. This result indicate that it is necessary for the high expression of MTG to change the codon of MTG gene into a codon for E. coli and also to multi-copy the plasmid.

Table 1

	Strain	MTG expression
5	pUCTRPM TG-02(+)/JM109	+++
	pUCTRPM TG-02(-)/JM109	+++
	pUCTRPM TG-00(+)/JM109	+
	pUCTRPM TG-00(-)/JM109	+
	pTRPMTG-02/JM109	+
	pTRPMTG-01/JM109	+
10	pTRPMTG-00/JM109	-
	pUC19/JM109	-

+++ : at least 300 mg/l

+ : 5 mg/l or below

- : no expression

<8> Analysis of N-terminal amino acid of expressed MTG:

The N-terminal amino acid residue of the protein inclusion bodies of expressed MTG was analyzed to find that about 60 % of the sequence of N-terminal was methionine residue and about 40 % thereof was formylmethionine residue. (Formyl)methionine residue corresponding to the initiation codon was removed by a technical idea described below.

<9> Deletion of N-terminal aspartic acid residue of MTG:

A base sequence corresponding to aspartic acid residue (the N-terminal of MTG) was deleted by PCR using pUCN216 containing 216 bases as the template. pUCN216 is a plasmid obtained by cloning about 216

bp's containing ClaI-HpaI fragment of N-terminal of MTG in EcoRI/HindIII site of pUC19. pF01 (SEQ ID No. 56) and pR01 (SEQ ID No. 57) are primers each having a sequence in the vector. pDELD (SEQ ID No. 58) is that obtained by deleting a base sequence corresponding to Asp residue. pHd01 (SEQ ID No. 59) is that obtained by replacing C with G not to include HindIII site. pF01 and pDELD are sense primers and pR01 and pHd01 are antisense primers.

5 35 cycles of PCR of a combination of pF01 and pHd01, and a combination of pELD and pR01 for pUCN216 was conducted at 94 °C for 30 seconds, at 55°C for one minute and at 72 °C for two minutes. Each PCR product was extracted with phenol/chloroform, precipitated with ethanol and dissolved in 100 μ l of H₂O.

10 1 μ l of each of the PCR products was taken, and they were mixed together. After the heat denaturation at 94 °C for 10 minutes, 35 cycles of PCR of a combination of pF01 and pHd01 was conducted at 94°C for 30 seconds, at 55°C for one minute and at 72 °C for two minutes.

15 The second PCR product was extracted with phenol/chloroform, precipitated with ethanol, and treated with HindIII and EcoRI. After pUC19 subcloning, pUCN216D was obtained (Fig. 5). The sequence of the obtained pUCN216D was confirmed to be the intended one.

20 <10> Construction of the plasmid encoding for MTG which lacks N-terminal aspartic acid:

25 Eco0109I/HpaI fragment (small) of pUCN216D was combined with Eco0109I/HpaI fragment (large) of pUCB1-1 (plasmid obtained by cloning HpaII/BglIII fragment of MTG gene in EcoRI/HindIII site of pUC19) to obtain pUCNB1-2D. Further, ClaI/BglIII fragment (small) of pUCNB1-2D was

combined with ClaI/B/BglIII fragment (large) of pUCTRPMTG-02(+) which
is a plasmid of high MTG expression to obtain pUC TRPMTG(+)D2, the
expression plasmid of MTG which lacks N-terminal aspartic acid (Fig. 6).
As a result, a plasmid containing MTG gene lacking GAI corresponding to
5 aspartic acid residue as shown in Fig. 7 was obtained.

<11> Expression of the plasmid encoding for MTG which lacks N-terminal
aspartic acid:

E. coli JM109 transformed with pUCTRPMTG(+)D2 was cultured by
shaking in 3 ml of 2xYT medium containing 150 μ g/ml of ampicillin at 37
10 °C for ten hours (pre-culture). 0.5 ml of the culture suspension was
added to 50 ml of 2xYT medium containing 150 μ g/ml of ampicillin, and
the shaking culture was conducted at 37 °C for 20 hours. The cells were
broken by the ultrasonic disintegration. The results of the dyeing
with Coomassie Brilliant Blue dyeing and Western blotting with mouse
15 antiMTG antibody of the thus obtained supernatant liquid and precipitate
indicated that MTG protein lacking N-terminal aspartic acid residue was
detected in the precipitate obtained by the ultrasonic disintegration,
namely in the insoluble fraction. This fact suggests that MTG protein
lacking N-terminal aspartic acid residue was accumulated as protein
20 inclusion bodies in the cells.

The N-terminal amino acid sequence of the protein inclusion
bodies was analyzed to find that about 90 % thereof was serine as shown
in Fig. 8.

The results of the analysis of N-terminal amino acids of
25 expressed MTG obtained in <8> and <11> were compared with each other as
shown in Table 2. It was found that by deleting the N-terminal

aspartic acid residue from MTG, the initiation methionine added to the N-terminal of the expressed MTG was efficiently removed.

Table 2

5	Strain	N-terminal amino acid			
		f-Met	Met	Asp	Ser
pUCTRPMTG-02(+)/JM109		40 %	60 %	N.D.	
pUCTRPMTG(+)D2/JM109		N.D.	10 %	-	90 %

10 <12> Solubilization of MTG inclusion bodies lacking N-terminal aspartic acid residue, renaturation of activity and determination of specific activity:

15 MTG inclusion bodies lacking aspartic acid was partially purified by repeating the centrifugation several times, and then dissolved in 8 M urea [50 mM phosphate buffer (pH 5.5)] to obtain the 2 mg/ml solution. Precipitates were removed from the solution by the centrifugation and the solution was diluted to a concentration of 0.5 M urea with 50 mM phosphate buffer (pH 5.5). The diluted solution was further dialyzed with 50 mM phosphate buffer (pH 5.5) to remove urea.
20 According to Mono S column test, the peak having TG activity was eluted when NaCl concentration was in the range of 100 to 150 mM. The specific activity of the fraction was determined by the hydroxamate method to find that the specific activity of the aspartic acid residue-lacking MTG was about 30 U/mg. This is equal to the specific activity of natural MT G. It is thus apparent that the lack of aspartic acid residue exerts no influence on the specific activity.
25

SEQUENCE LISTING

INFORMATION FOR SEQ ID NO:1:

SEQUENCE CHARACTERISTICS:

LENGTH:331

TYPE:amino acid

TOPOLOGY:linear

MOLECULE TYPE:peptide

SEQUENCE DESCRIPTION: SEQ ID NO:1

Asp Ser Asp Asp Arg Val Thr Pro Pro Ala Glu Pro Leu Asp Arg Met

1 5 10 15

Pro Asp Pro Tyr Arg Pro Ser Tyr Gly Arg Ala Glu Thr Val Val Asn

20 25 30

Asn Tyr Ile Arg Lys Trp Gln Gln Val Tyr Ser His Arg Asp Gly Arg

35 40 45

Lys Gln Gln Met Thr Glu Glu Gln Arg Glu Trp Leu Ser Tyr Gly Cys

50 55 60

Val Gly Val Thr Trp Val Asn Ser Gly Gln Tyr Pro Thr Asn Arg Leu

65 70 75 80

Ala Phe Ala Ser Phe Asp Glu Asp Arg Phe Lys Asn Glu Leu Lys Asn

85 90 95

Gly Arg Pro Arg Ser Gly Glu Thr Arg Ala Glu Phe Glu Gly Arg Val

100 105 110

Ala Lys Glu Ser Phe Asp Glu Glu Lys Gly Phe Gln Arg Ala Arg Glu

115 120 125

Val Ala Ser Val Met Asn Arg Ala Leu Glu Asn Ala His Asp Glu Ser

130 135 140

Ala Tyr Leu Asp Asn Leu Lys Lys Glu Leu Ala Asn Gly Asn Asp Ala

145 150 155 160

Leu Arg Asn Glu Asp Ala Arg Ser Pro Phe Tyr Ser Ala Leu Arg Asn

165 170 175

Thr Pro Ser Phe Lys Glu Arg Asn Gly Gly Asn His Asp Pro Ser Arg

180 185 190

Met Lys Ala Val Ile Tyr Ser Lys His Phe Trp Ser Gly Gln Asp Arg

195 200 205

Ser Ser Ser Ala Asp Lys Arg Lys Tyr Gly Asp Pro Asp Ala Phe Arg

210 215 220

Pro Ala Pro Gly Thr Gly Leu Val Asp Met Ser Arg Asp Arg Asn Ile

225 230 235 240

Pro Arg Ser Pro Thr Ser Pro Gly Glu Gly Phe Val Asn Phe Asp Tyr

245 250 255

Gly Trp Phe Gly Ala Gln Thr Glu Ala Asp Ala Asp Lys Thr Val Trp

260

265

270

Thr His Gly Asn His Tyr His Ala Pro Asn Gly Ser Leu Gly Ala Met

275

280

285

His Val Tyr Glu Ser Lys Phe Arg Asn Trp Ser Glu Gly Tyr Ser Asp

290

295

300

Phe Asp Arg Gly Ala Tyr Val Ile Thr Phe Ile Pro Lys Ser Trp Asn

305

310

315

320

Thr Ala Pro Asp Lys Val Lys Gln Gly Trp Pro

325

330

INFORMATION FOR SEQ ID NO:2:

SEQUENCE CHARACTERISTICS:

LENGTH: 993

TYPE: nucleic acid

STRANDEDNESS: double

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

FEATURE

FEATURE KEY: CDS

LOCATION: 1..993

IDENTIFICATION METHOD : S

SEQUENCE DESCRIPTION: SEQ ID NO:2

GAT TCT GAC GAT CGT GTT ACT CCA CCA GCT GAA CCA CTG GAT CGT ATG

48

Asp Ser Asp Asp Arg Val Thr Pro Pro Ala Glu Pro Leu Asp Arg Met

1

5

10

15

CCA GAT CCA TAT CGT CCA TCT TAT GGT CGT GCT GAA ACT GTT GTT AAT

96

Pro Asp Pro Tyr Arg Pro Ser Tyr Gly Arg Ala Glu Thr Val Val Asn

20

25

30

AAT TAT ATT CGT AAA TGG CAA CAA GTT TAT TCT CAT CGT GAT GGT CGT

144

Asn Tyr Ile Arg Lys Trp Gln Gln Val Tyr Ser His Arg Asp Gly Arg

35

40

45

AAA CAA CAA ATG ACT GAA GAA CAA CGT GAA TGG CTG TCT TAT GGT TGC

192

Lys Gln Gln Met Thr Glu Glu Gln Arg Glu Trp Leu Ser Tyr Gly Cys

50

55

60

GTT GGT GTT ACT TGG GTT AAC TCT GGT CAG TAT CCG ACT AAC CGT CTG

240

Val Gly Val Thr Trp Val Asn Ser Gly Gln Tyr Pro Thr Asn Arg Leu

65

70

75

80

GCA TTC GCT TCC TTC GAT GAA GAT CGT TTC AAG AAC GAA CTG AAG AAC

288

Ala Phe Ala Ser Phe Asp Glu Asp Arg Phe Lys Asn Glu Leu Lys Asn

85

90

95

GGT CGT CCG CGT TCT GGT GAA ACT CGT GCT GAA TTC GAA GGT CGT GTT
336

Gly Arg Pro Arg Ser Gly Glu Thr Arg Ala Glu Phe Glu Gly Arg Val
100 105 110

GCT AAG GAA TCC TTC GAT GAA GAG AAA GGC TTC CAG CGT GCT CGT GAA
384

Ala Lys Glu Ser Phe Asp Glu Glu Lys Gly Phe Gln Arg Ala Arg Glu
115 120 125

GTT GCT TCT GTT ATG AAC CGT GCT CTA GAG AAC GCT CAT GAT GAA TCT
432

Val Ala Ser Val Met Asn Arg Ala Leu Glu Asn Ala His Asp Glu Ser
130 135 140

GCT TAC CTG GAT AAC CTG AAG AAG GAA CTG GCT AAC GGT AAC GAT GCT
480

Ala Tyr Leu Asp Asn Leu Lys Lys Glu Leu Ala Asn Gly Asn Asp Ala
145 150 155 160

CTG CGT AAC GAA GAT GCT CGT TCT CCG TTC TAC TCT GCT CTG CGT AAC
528

Leu Arg Asn Glu Asp Ala Arg Ser Pro Phe Tyr Ser Ala Leu Arg Asn
165 170 175

ACT CCG TCC TTC AAA GAA CGT AAC GGT GGT AAC CAT GAT CCG TCT CGT
576

Thr Pro Ser Phe Lys Glu Arg Asn Gly Gly Asn His Asp Pro Ser Arg

180 185 190

ATG AAA GCT GTT ATC TAC TCT AAA CAT TTC TGG TCT GGT CAG GAT AGA
624
Met Lys Ala Val Ile Tyr Ser Lys His Phe Trp Ser Gly Gln Asp Arg
195 200 205

TCT TCT TCT GCT GAT AAA CGT AAA TAC GGT GAT CCG GAT GCA TTC CGT
672
Ser Ser Ser Ala Asp Lys Arg Lys Tyr Gly Asp Pro Asp Ala Phe Arg
210 215 220

CCG GCT CCG GGT ACT GGT CTG GTA GAC ATG TCT CGT GAT CGT AAC ATC
720
Pro Ala Pro Gly Thr Gly Leu Val Asp Met Ser Arg Asp Arg Asn Ile
225 230 235 240

CCG CGT TCT CCG ACT TCT CCG GGT GAA GGC TTC GTT AAC TTC GAT TAC
768
Pro Arg Ser Pro Thr Ser Pro Gly Glu Gly Phe Val Asn Phe Asp Tyr
245 250 255

GGT TGG TTC GGT GCT CAG ACT GAA GCT GAT GCT GAT AAG ACT GTA TGG
816
Gly Trp Phe Gly Ala Gln Thr Glu Ala Asp Ala Asp Lys Thr Val Trp
260 265 270

ACC CAT GGT AAC CAT TAC CAT GCT CCG AAC GGT TCT CTG GGT GCT ATG
864

Thr His Gly Asn His Tyr His Ala Pro Asn Gly Ser Leu Gly Ala Met

275

280

285

CAT GTA TAC GAA TCT AAA TTC CGT AAC TGG TCT GAA GGT TAC TCT GAC

912

His Val Tyr Glu Ser Lys Phe Arg Asn Trp Ser Glu Gly Tyr Ser Asp

290

295

300

TTC GAT CGT GGT GCT TAC GTT ATC ACC TTC ATT CCG AAA TCT TGG AAC

960

Phe Asp Arg Gly Ala Tyr Val Ile Thr Phe Ile Pro Lys Ser Trp Asn

305

310

315

320

ACT GCT CCG GAC AAA GTT AAA CAG GGT TGG CCG

993

Thr Ala Pro Asp Lys Val Lys Gln Gly Trp Pro

325

330

INFORMATION FOR SEQ ID NO:3:

SEQUENCE CHARACTERISTICS:

LENGTH: 1518

TYPE: nucleic acid

STRANDEDNESS: double

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

FEATURE

FEATURE KEY:CDS

LOCATION: 87..1082

IDENTIFICATION METHOD:S

SEQUENCE DESCRIPTION: SEQ ID NO:3

TTCCCCCTGTT GACAATTAAT CATCGAACTA GTTAACTAGT ACGCAAGTTC ACGTAAAAAG

60

GGTATCGATT AGTAAGGAGG TTTAAA ATG GAT TCT GAC GAT CGT GTT ACT CCA

113

Met Asp Ser Asp Asp Arg Val Thr Pro

1

5

CCA GCT GAA CCA CTG GAT CGT ATG CCA GAT CCA TAT CGT CCA TCT TAT

161

Pro Ala Glu Pro Leu Asp Arg Met Pro Asp Pro Tyr Arg Pro Ser Tyr

10

15

20

25

GGT CGT GCT GAA ACT GTT GTT AAT AAT TAT ATT CGT AAA TGG CAA CAA

209

Gly Arg Ala Glu Thr Val Val Asn Asn Tyr Ile Arg Lys Trp Gln Gln

30

35

40

GTT TAT TCT CAT CGT GAT GGT CGT AAA CAA CAA ATG ACT GAA GAA CAA

257

Val Tyr Ser His Arg Asp Gly Arg Lys Gln Gln Met Thr Glu Glu Gln

45

50

55

CGT GAA TGG CTG TCT TAT GGT TGC GTT GGT GTT ACT TGG GTT AAC TCT

305

Arg Glu Trp Leu Ser Tyr Gly Cys Val Gly Val Thr Trp Val Asn Ser

60

65

70

GGT CAG TAT CCG ACT AAC CGT CTG GCA TTC GCT TCC TTC GAT GAA GAT

353

Gly Gln Tyr Pro Thr Asn Arg Leu Ala Phe Ala Ser Phe Asp Glu Asp

75

80

85

CGT TTC AAG AAC GAA CTG AAG AAC GGT CGT CCG CGT TCT GGT GAA ACT

401

Arg Phe Lys Asn Glu Leu Lys Asn Gly Arg Pro Arg Ser Gly Glu Thr

90

95

100

105

CGT GCT GAA TTC GAA GGT CGT GTT GCT AAG GAA TCC TTC GAT GAA GAG

449

Arg Ala Glu Phe Glu Gly Arg Val Ala Lys Glu Ser Phe Asp Glu Glu

110

115

120

AAA GGC TTC CAG CGT GCT CGT GAA GTT GCT TCT GTT ATG AAC CGT GCT

497

Lys Gly Phe Gln Arg Ala Arg Glu Val Ala Ser Val Met Asn Arg Ala

125

130

135

CTA GAG AAC GCT CAT GAT GAA TCT GCT TAC CTG GAT AAC CTG AAG AAG

545

Leu Glu Asn Ala His Asp Glu Ser Ala Tyr Leu Asp Asn Leu Lys Lys

140

145

150

GAA CTG GCT AAC GGT AAC GAT GCT CTG CGT AAC GAA GAT GCT CGT TCT

593

Glu Leu Ala Asn Gly Asn Asp Ala Leu Arg Asn Glu Asp Ala Arg Ser

155 160 165

CCG TTC TAC TCT GCT CTG CGT AAC ACT CCG TCC TTC AAA GAA CGT AAC
641

Pro Phe Tyr Ser Ala Leu Arg Asn Thr Pro Ser Phe Lys Glu Arg Asn
170 175 180 185

GGT GGT AAC CAT GAT CCG TCT CGT ATG AAA GCT GTT ATC TAC TCT AAA
689

Gly Gly Asn His Asp Pro Ser Arg Met Lys Ala Val Ile Tyr Ser Lys
190 195 200

CAT TTC TGG TCT GGT CAG GAT AGA TCT TCT TCT GCT GAT AAA CGT AAA
737

His Phe Trp Ser Gly Gln Asp Arg Ser Ser Ser Ala Asp Lys Arg Lys
205 210 215

TAC GGT GAT CCG GAT GCA TTC CGT CCG GCT CCG GGT ACT GGT CTG GTA
785

Tyr Gly Asp Pro Asp Ala Phe Arg Pro Ala Pro Gly Thr Gly Leu Val
220 225 230

GAC ATG TCT CGT GAT CGT AAC ATC CCG CGT TCT CCG ACT TCT CCG GGT
833

Asp Met Ser Arg Asp Arg Asn Ile Pro Arg Ser Pro Thr Ser Pro Gly
235 240 245

GAA GGC TTC GTT AAC TTC GAT TAC GGT TGG TTC GGT GCT CAG ACT GAA
881

Glu Gly Phe Val Asn Phe Asp Tyr Gly Trp Phe Gly Ala Gln Thr Glu
250 255 260 265

GCT GAT GCT GAT AAG ACT GTA TGG ACC CAT GGT AAC CAT TAC CAT GCT
929

Ala Asp Ala Asp Lys Thr Val Trp Thr His Gly Asn His Tyr His Ala
270 275 280

CCG AAC GGT TCT CTG GGT GCT ATG CAT GTA TAC GAA TCT AAA TTC CGT
977

Pro Asn Gly Ser Leu Gly Ala Met His Val Tyr Glu Ser Lys Phe Arg
285 290 295

AAC TGG TCT GAA GGT TAC TCT GAC TTC GAT CGT GGT GCT TAC GTT ATC
1025

Asn Trp Ser Glu Gly Tyr Ser Asp Phe Asp Arg Gly Ala Tyr Val Ile
300 305 310

ACC TTC ATT CCG AAA TCT TGG AAC ACT GCT CCG GAC AAA GTT AAA CAG
1073

Thr Phe Ile Pro Lys Ser Trp Asn Thr Ala Pro Asp Lys Val Lys Gln
315 320 325

GGT TGG CCG TAATGAAAGC TTGGATCTCT ATTACTGGA CTTCACACAG ACTAAAATAG
1131

Gly Trp Pro

330

ACATATCTTA TATTATGTGA TTTTGACAGA TTTCTAGAT GTGAGGTGGA GGTGATGTAT

1191

AAGGTAGATG ATGATCCTCT ACGCCGGACG CATCGTGGCC GGCATCACCG GCGCCACAGG

1251

TGCGGTTGCT GGCGCCTATA TCGCCGACAT CACCGATGGG GAAGATCGGG CTCGCCACTT

1311

CGGGCTCATG AGCGCTTGT TCGGCCTGGG TATGGTGGCA GGCCCCGTGG CGGGGGGACT

1371

GTTGGGCGCC ATCTCCTTGC ATGCACCATT CCTTGCGGGCG GCGGTGCTCA ACGGCCTCAA

1431

CCTACTACTG GGCTGCTTCC TAATGCAGGA GTGCATAAG GGAGAGCGTC GAGAGCCCGC

1491

CTAATGAGCG GGCTTTTT TCAGCTG

1518

INFORMATION FOR SEQ ID NO:4:

SEQUENCE CHARACTERISTICS:

LENGTH:39

TYPE:nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:4

AATTCATCGA TTAGTAAGGA GGTTAAAAT GGATTCTGA

39

INFORMATION FOR SEQ ID NO:5:

SEQUENCE CHARACTERISTICS:

LENGTH: 41

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:5

CGATCGTCAG AATCCATTAAACCTCCTT ACTAATCGAT G

41

INFORMATION FOR SEQ ID NO:6:

SEQUENCE CHARACTERISTICS:

LENGTH: 41

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:6

CGATCGTGTT ACTCCACCAG CTGAACCACT GGATCGTATG C

41

INFORMATION FOR SEQ ID NO:7:

SEQUENCE CHARACTERISTICS:

LENGTH: 41

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:7

GATCTGGCAT ACGATCCAGT GGTCAGCTG GTGGAGTAAC A

41

INFORMATION FOR SEQ ID NO:8:

SEQUENCE CHARACTERISTICS:

LENGTH:41

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:8

CAGATCCATA TCGTCCATCT TATGGTCGTG CTGAAACTGT T

41

INFORMATION FOR SEQ ID NO:9:

SEQUENCE CHARACTERISTICS:

LENGTH:41

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

40

SEQUENCE DESCRIPTION: SEQ ID NO:9

ATTAACAAACA GTTTCAGCAC GACCATAAGA TGGACGATAT G

41

INFORMATION FOR SEQ ID NO:10:

SEQUENCE CHARACTERISTICS:

LENGTH:41

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:10

GTTAATAATT ATATTCGTAA ATGGCAACAA GTTTATTCTC A

41

INFORMATION FOR SEQ ID NO:11:

SEQUENCE CHARACTERISTICS:

LENGTH:41

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:11

TCACGATGAG AATAAACTTG TTGCCATTAA CGAATATAAT T

41

INFORMATION FOR SEQ ID NO:12:

SEQUENCE CHARACTERISTICS:

LENGTH:41

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:12

TCGTGATGGT CGTAAACAAAC AAATGACTGA AGAACAAACGT G

41

INFORMATION FOR SEQ ID NO:13:

SEQUENCE CHARACTERISTICS:

LENGTH:41

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:13

GCCATTACG TTGTTCTTCA GTCATTTGTT GTTTACGACC A

41

INFORMATION FOR SEQ ID NO:14:

SEQUENCE CHARACTERISTICS:

LENGTH:42

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:14

AATGGCTGTC TTATGGTTGC GTTGGTGTAA CTTGGGTTAA CA

42

INFORMATION FOR SEQ ID NO:15:

SEQUENCE CHARACTERISTICS:

LENGTH:40

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:15

AGCTTGTAA CCCAAGTAAC ACCAACGCAA CCATAAGACA

40

INFORMATION FOR SEQ ID NO:16:

SEQUENCE CHARACTERISTICS:

LENGTH:38

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:16

AATTCGTTAA CTCTGGTCAG TATCCGACTA ACCGTCTG

38

INFORMATION FOR SEQ ID NO:17:

SEQUENCE CHARACTERISTICS:

LENGTH:41

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:17

CGAATGCCAG ACGGTTAGTC GGATACTGAC CAGAGTTAAC G

41

INFORMATION FOR SEQ ID NO:18:

SEQUENCE CHARACTERISTICS:

LENGTH:49

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:18

GCATTCGCTT CCTTCGATGA AGATCGTTTC AAGAACGAAC TGAAGAACG

49

INFORMATION FOR SEQ ID NO:19:

SEQUENCE CHARACTERISTICS:

LENGTH:49

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:19

GGACGACCGT TCTTCAGTTC GTTCTTGAAA CGATCTTCAT CGAAGGAAG

49

INFORMATION FOR SEQ ID NO:20:

SEQUENCE CHARACTERISTICS:

LENGTH:35

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:20

GTCGTCCGCG TTCTGGTGAA ACTCGTGCTG AATTC

INFORMATION FOR SEQ ID NO:21:

SEQUENCE CHARACTERISTICS:

LENGTH:35

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:21

GACCTTCGAA TTCAGCACGA GTTTCACCAG AACGC

35

INFORMATION FOR SEQ ID NO:22:

SEQUENCE CHARACTERISTICS:

LENGTH:48

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:22

GAAGGTCGTG TTGCTAAGGA ATCCTTCGAT GAAGAGAAAG GCTTCCAG

48

INFORMATION FOR SEQ ID NO:23:

SEQUENCE CHARACTERISTICS:

LENGTH: 48

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:23

GAGCACGCTG GAAGCCTTTC TCTTCATCGA AGGATTCCCTT AGCAACAC

48

INFORMATION FOR SEQ ID NO:24:

SEQUENCE CHARACTERISTICS:

LENGTH: 42

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:24

CGTGCTCGTG AAGTTGCTTC TGTTATGAAC CGTGCTCTAG AA

42

INFORMATION FOR SEQ ID NO:25:

SEQUENCE CHARACTERISTICS:

LENGTH: 39

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:25

AGCTTTCTAG AGCACGGTTC ATAACAGAAG CAACTTCAC

39

INFORMATION FOR SEQ ID NO:26:

SEQUENCE CHARACTERISTICS:

LENGTH:45

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:26

AATTCTCTAG AGAACGCTCA TGATGAATCT GCTTACCTGG ATAAC

45

INFORMATION FOR SEQ ID NO:27:

SEQUENCE CHARACTERISTICS:

LENGTH:50

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

4 8

SEQUENCE DESCRIPTION: SEQ ID NO:27

CTTCTTCAGG TTATCCAGGT AAGCAGATTC ATCATGAGCG TTCTCTAGAG

50

INFORMATION FOR SEQ ID NO:28:

SEQUENCE CHARACTERISTICS:

LENGTH:49

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:28

CTGAAGAAGG AACTGGCTAA CGGTAACGAT GCTCTGCGTA ACGAAGATG

49

INFORMATION FOR SEQ ID NO:29:

SEQUENCE CHARACTERISTICS:

LENGTH:49

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:29

GAGAACGAGC ATCTTCGTTA CGCAGAGCAT CGTTACCGTT AGCCAGTTC

INFORMATION FOR SEQ ID NO:30

SEQUENCE CHARACTERISTICS:

LENGTH:40

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:30

CTCGTTCTCC GTTCTACTCT GCTCTGCGTA ACACCTCCGTC

INFORMATION FOR SEQ ID NO:31:

SEQUENCE CHARACTERISTICS:

LENGTH:39

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:31

CTTTGAAGGA CGGAGTGTTA CGCAGAGCAG AGTAGAACG

INFORMATION FOR SEQ ID NO:32:

SEQUENCE CHARACTERISTICS:

LENGTH: 47

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:32

CTTCAAAGAA CGTAACGGTG GTAACCATGA TCCGTCTCGT ATGAAAG

47

INFORMATION FOR SEQ ID NO:33:

SEQUENCE CHARACTERISTICS:

LENGTH: 47

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:33

GATAAACAGCT TTCATACGAG ACGGATCATG GTTACCACCG TTACGTT

47

INFORMATION FOR SEQ ID NO:34:

SEQUENCE CHARACTERISTICS:

LENGTH: 45

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:34

CTGTTATCTA CTCTAACAT TTCTGGTCTG GTCAGGATAG ATCTA

45

INFORMATION FOR SEQ ID NO:35:

SEQUENCE CHARACTERISTICS:

LENGTH:41

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:35

AGCTTAGATC TATCCTGACC AGACCAGAAA TGTTTAGAGT A

41

INFORMATION FOR SEQ ID NO:36:

SEQUENCE CHARACTERISTICS:

LENGTH:42

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:36

AATTCAGATC TTCTTCTGCT GATAAACGTA AATACGGTGA TC

42

INFORMATION FOR SEQ ID NO:37:

SEQUENCE CHARACTERISTICS:

LENGTH: 44

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:37

CATCCGGATC ACCGTATTTA CGTTTATCAG CAGAAGAAGA TCTG

44

INFORMATION FOR SEQ ID NO:38:

SEQUENCE CHARACTERISTICS:

LENGTH: 48

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:38

CGGATGCATT CCGTCCGGCT CCGGGTACTG GTCTGGTAGA CATGTCTC

48

INFORMATION FOR SEQ ID NO:39:

SEQUENCE CHARACTERISTICS:

LENGTH: 48

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:39

GATCACGAGA CATGTCTACC AGACCAGTAC CCGGAGCCGG ACGGAATG

48

INFORMATION FOR SEQ ID NO:40:

SEQUENCE CHARACTERISTICS:

LENGTH: 35

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:40

GTGATCGTAA CATCCCGCGT TCTCCGACTT CTCCG

35

INFORMATION FOR SEQ ID NO:41:

SEQUENCE CHARACTERISTICS:

LENGTH:36

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:41

CTTCACCCGG AGAACGTCGGA GAACGCGGGGA TGTTAC

36

INFORMATION FOR SEQ ID NO:42:

SEQUENCE CHARACTERISTICS:

LENGTH:40

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:42

GGTGAAGGCT TCGTTAACTT CGATTACGGT TGGTTCGGTG

40

INFORMATION FOR SEQ ID NO:43:

SEQUENCE CHARACTERISTICS:

LENGTH:40

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:43

GTCTGAGCAC CGAACCAACC GTAATCGAAG TTAACGAAGC

40

INFORMATION FOR SEQ ID NO:44

SEQUENCE CHARACTERISTICS:

LENGTH:44

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:44

CTCAGACTGA AGCTGATGCT GATAAGACTG TATGGACCCA TGGA

44

INFORMATION FOR SEQ ID NO:45

SEQUENCE CHARACTERISTICS:

LENGTH:41

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:45

AGCTTCCATG GGTCCATACA GTCTTATCAG CATCAGCTTC A

41

INFORMATION FOR SEQ ID NO:46

SEQUENCE CHARACTERISTICS:

LENGTH:39

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:46

AATTCCCATG GTAACCATTA CCATGCTCCG AACGGTTCT

39

INFORMATION FOR SEQ ID NO:47

SEQUENCE CHARACTERISTICS:

LENGTH:42

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:47

CACCCAGAGA ACCGTTCGGA GCATGGTAAT GGTTACCATG GG

42

INFORMATION FOR SEQ ID NO:48

SEQUENCE CHARACTERISTICS:

LENGTH:41

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:48

CTGGGTGCTA TGCATGTATA CGAATCTAAA TTCCGTAAC G

41

INFORMATION FOR SEQ ID NO:49

SEQUENCE CHARACTERISTICS:

LENGTH:42

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:49

CTTCAGACCA GTTACGGAAT TTAGATTCTGT ATACATGCAT AG

42

INFORMATION FOR SEQ ID NO:50

SEQUENCE CHARACTERISTICS:

LENGTH:37

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:50

GTCTGAAGGT TACTCTGACT TCGATCGTGG TGCTTAC

37

INFORMATION FOR SEQ ID NO:51

SEQUENCE CHARACTERISTICS:

LENGTH:37

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:51

GTGATAACGT AAGCACCACG ATCGAAGTCA GAGTAAC

37

INFORMATION FOR SEQ ID NO:52

SEQUENCE CHARACTERISTICS:

LENGTH:38

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:52

GTTATCACCT TCATTCCGAA ATCTTGGAAC ACTGCTCC

38

INFORMATION FOR SEQ ID NO:53

SEQUENCE CHARACTERISTICS:

LENGTH:38

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:53

CTTTGTCCGG AGCAGTGTTC CAAGATTCG GAATGAAG

38

INFORMATION FOR SEQ ID NO:54

SEQUENCE CHARACTERISTICS:

LENGTH:38

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:54

GGACAAAGTT AACAGGGTT GGCGTAATG AAAGCTTA

38

INFORMATION FOR SEQ ID NO:55

SEQUENCE CHARACTERISTICS:

LENGTH:34

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:55

AGCTTAAGCT TTCATTACGG CCAACCCTGT TTAA

34

INFORMATION FOR SEQ ID NO:56

SEQUENCE CHARACTERISTICS:

LENGTH:20

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

61

SEQUENCE DESCRIPTION: SEQ ID NO:56

TTTTCCCAGT CACGACGTTG

20

INFORMATION FOR SEQ ID NO:57

SEQUENCE CHARACTERISTICS:

LENGTH:21

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:57

CAGGAAACAG CTATGACCAT G

21

INFORMATION FOR SEQ ID NO:58

SEQUENCE CHARACTERISTICS:

LENGTH:36

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:58

TAAGGAGGTT TAAAATGTCT GACGATCGTG TTACTC

INFORMATION FOR SEQ ID NO:59

SEQUENCE CHARACTERISTICS:

LENGTH:21

TYPE: nucleic acid

STRANDEDNESS:single

TOPOLOGY: linear

MOLECULE TYPE:other nucleic acid synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO:59

TACGCCAAGG TTGTTAACCC A

21